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Expression of Retinoid-Responsive Genes Occurs in Colorectal Carcinoma-Derived Cells Irrespective of the Presence of Resistance to All-Trans Retinoic Acid

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Background and Objectives: Retinoids are metabolized in human intestinal epithelial cells to all-trans retinoic acid; however, it is unknown whether these cells express retinoid receptors, and whether sensitivity or resistance to the hormone is associated with a particular pattern of expression of retinoid-responsive genes.

Methods: Northern blot analysis and reverse transcriptase polymerase chain reaction (RT-PCR) were used to identify mRNAs for retinoid receptors. Both Relative RT-PCR and transfection of retinoid-inducible plasmid were applied to test functionality of the pathway in a model system for colorectal carcinoma progression (primary SW480, all-trans retinoic acid-sensitive cells vs. metastatic SW620, -insensitive cells).

Results: Three colorectal carcinoma-derived cell lines were inhibited by the hormone. Retinoic acid receptor type α (hRARα) and retinoid X receptor type α (hRXRα) mRNAs were detected in normal enterocytes, colonocytes, and in all colorectal carcinoma-derived cells studied. Primary carcinomas and metastatic lesions expressed high amounts of hRARα receptor protein, showing no simple correlation between the amounts of mRNA and receptor protein. No pattern of expression of the retinoid-responsive genes was associated with sensitivity or resistance to the retinoid. Expression of the genes occurred irrespective of resistance to the hormone or inactivity of the pathway.

Abbreviations: atRA, all-trans retinoic acid; CAT, chloramphenicol acetyltransferase; CRBP I , cellular retinol binding protein type I; CRABP I, II, cellular retinoic acid binding protein types I and II; EGF-R, epidermal growth factor receptor; hRAR, retinoic acid receptor; hRXR, retinoid X receptor; MK, angiogenic cytokine; RARE, retinoic acid responsive element; TG, transglutaminase; DEPC, diethylpyrocarbonate; DTT, dithiothreitol; dNTP; deoxynucleotidetriphosphate; PMSF, phenylmethylsulfonyl fluoride.

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Conclusions: Colonocytes possess a molecular system for transduction of the retinoid signal. All-trans retinoic acid modifies gene expression and inhibits proliferation of these cells. Therefore, retinoids are likely to be effective in chemoprevention of colorectal carcinoma.

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KEY WORDS: retinoids; retinoid receptors; retinoid-responsive genes; complexity; colorectal neoplasms

INTRODUCTION

The traditional view of retinoids, vitamin A and its derivatives, either as growth factors (vitamin A) or a chromophore involved in light absorption during the vision process (11-cis-retinal), changed when their activity in modifying expression of several genes important for cellular proliferation and differentiation was discovered [1, 2]. This finding provided a background for chemoprevention, a relatively new approach to cancer control, in which cellular differentiation, proliferation, or induction of apoptotic death are manipulated in initiated cells progressing toward malignancy, transformed cells, and adjacent cells comprising the epithelial and stromal field [3].

All-trans retinoic acid, actively synthesized in various tissues as a result of enzymatic transformation of vitamin A and β-carotene [4, 5], was found to be one of the most biologically active retinoids in exerting an antitumorigenic effect in a variety of transformed epithelial cells [2, 3]. The signaling pathway involves binding to specific high-affinity nuclear receptors [1, 6]. The retinoid receptors are classified into the retinoic acid receptor (hRAR) and retinoid X receptor (hRXR) subfamilies. The hRAR family is comprised of α, β, and γ subtypes [7, 8] while only α and β subtypes have been identified for the hRXR subfamily to date [9–11]. The highest affinity ligands for the hRAR and hRXR subfamilies are all-trans retinoic acid and 9-cis retinoic acids, respectively [12]. The hRAR and hRXR proteins form heterodimers that bind much more efficiently to DNA retinoid-responsive elements than do hRAR-hRAR homodimers [11]. The ligand-receptor complexes bind to retinoid-responsive elements on gene promoters and modulate gene transcription in a ligand- and dose-dependent manner. Expression of the retinoid-responsive genes is believed to determine cellular sensitivity to retinoids [1].

Retinoids are also bound in the cytoplasm by a high-affinity binding protein, cellular retinol binding protein type I (CRBP I), which binds retinol, and cellular retinoic acid binding protein types I and II (CRABP I and CRABP II), which bind all-trans retinoic acid [13–16]. These latter two proteins seem to modulate the activity of all-trans retinoic acid, at least in part, by inhibiting interaction with the nuclear receptors.

Vitamin A and β-carotene are absorbed and enzymatically metabolized in enterocytes in ileum [17]. Recently, the enzymatic systems converting vitamin A to the biologically active all trans-retinoic acid were discovered in the human colon carcinoma-derived cells, Caco 2 [18]. Epidemiological data relating vitamin A and colon carcinoma are contradictory; some investigators reported that intestinal mucosa was protected by retinoids during colorectal tumorigenesis [19] while others reported no correlation between risk of colorectal carcinoma and the amount of vitamin A in the diet [20, 21]. In animal studies of experimental tumorigenesis, retinoids unequivocally decreased the frequency of colorectal carcinomas [22, 23]. No chemoprevention trials of retinoids in human colorectal carcinoma have been reported; however, it was demonstrated that retinoids had clear antiproliferative effect on some established human colorectal carcinoma cell lines [24–26]. Given the finding of the retinoid-metabolizing enzymes in Caco-2 cells, the question arises whether the all-trans retinoic acid signaling pathway is defective and whether loss of responsiveness to all-trans retinoic acid plays a role in human colorectal tumorigenesis. Although the presence of retinoic acid-binding activity in the cytosol of colonic mucosa and tumor specimens has been demonstrated [27], no reports have appeared concerning the presence of elements of the signaling pathway in human colonocytes. Also, no data exist concerning when in the process of tumorigenesis retinoid sensitivity might be lost or what mechanisms for loss of sensitivity would be responsible.

This study was undertaken to identify elements of the all-trans retinoic acid signaling pathway in intestinal epithelial cells, and test whether resistance or susceptibility to the proliferation-inhibiting activity of this retinoid was associated with a particular pattern of expression of retinoid-responsive genes. A series of seven colorectal carcinoma cell lines was surveyed to identify cell lines sensitive to proliferation inhibition by all-trans retinoic acid. The SW 480 cells derived from a primary tumor were sensitive to the hormone while the SW 620 cells derived from a lymph node metastasis from the same patient were resistant. Therefore, these cell types form an in vitro model of human colorectal carcinoma in which influences of retinoids on gene expression in relation to sensitivity in genetically related cells can be investigated. Northern blot analysis and RT-PCR were used to identify

mRNAs encoding hRARα and hRXRα receptor proteins. Expression of a series of retinoid-responsive genes—hRARα, hRARβ, CRBP I, CRABP I, and CRABP II [1], epidermal growth factor receptor (EGF-R)[1, 28], transglutaminase (TG)[29], angiogenic cytokine (MK) [30], as well as hRXRα, and hRXRβ was investigated in the model. Subsequently, both cell types were transfected with a plasmid containing the chloramphenicol acetyl transferase (CAT) gene under control of the retinoic acid responsive element (RARE) from the hRARβ gene and functionality of the pathway was evaluated. In addition, and for the first time, these results in vitro were compared to studies performed with clinical material, in which constitutive levels of retinoid receptor mRNAs and proteins were determined on cells derived from primary tumors and liver metastases, and enterocytes and colonocytes obtained from normal mucosa from the same patient.

MATERIALS AND METHODS

Surgical Specimens

Normal-appearing ileal and colonic mucosa was obtained from one patient with primary adenocarcinoma (HCC 111). Four primary tumor samples and four liver metasteses were also obtained. Tissue samples were rapidly transported from the operating room to the surgical pathology suite of the Cleveland Clinic Foundation and were immediately processed as described below. In some cases, corresponding samples from the same patient were obtained. With the case HCC 111 corresponding enterocytes, colonocytes and primary tumor cells were analyzed by Northern blot analysis or ordinary RT-PCR (not Relative RT-PCR) and the enhanced chemoluminescence (ECL) assay for hRARα receptor protein. Two liver metastases (HCC 134 and HCC 139) were analyzed by Northern blot analysis, ordinary RT-PCR and ECL assay. In the case of HCC 139, corresponding primary tumor was included in the ECL assay.

Cell Culture and Isolation of Intestinal Epithelial Cells

Established colorectal carcinoma cell lines, Caco 2, Colo 205, DLD 1, HT 29, SW 480, SW620, SW 837, SW 1463, representing different stages of the disease, were purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured in red phenol-free RPMI-1640 medium with 10% fetal calf serum (Gibco BRL, Gaithersburg, MD)[31]. For isolation of intestinal epithelial cells, mucosa was separated mechanically from the smooth muscle layer and cut into pieces 5 × 5 mm. The pieces were washed for 30 minutes in DTT/RPMI-1640 solution (1.5 mg/ml) at 37°C with shaking, followed by incubation three times for 30 minutes with Dispase (neutral protease from Bacillus polimyxa, Boehringer, Mannheim, Germany) dissolved in RPMI-1640 medium (3 mg/ml) with vigorous shaking (225 rpm). Red blood cells were lysed by incubation 15 minutes at room temperature in hypotonic solution (7 mg NH_4CO_3 and 700 mg NH_4Cl in 100 ml H_2O) followed by washing twice in cold PBS. The suspension contained 97% viable cells as demonstrated by staining with Trypan Blue. The cells were easily identified as epithelial cells on the basis of morphology and size. No red blood cells were found. Also, stromal cells were not present. Single lymphocytes were identified (1.8% ± 0.3%) as demonstrated by indirect immunostaining of the air-dried alcohol-fixed cellular smear using anti-keratin monoclonal antibody AE1/AE3 (Boehringer Mannheim, Germany), polyclonal antibodies directed to a desmin and a vimentin (Dako, Copenhagen, Denmark) and a monoclonal antibody directed to a leukocyte common antigen CD45RB (Dako, Carpinteria, CA)[32].

Effect of All-Trans Retinoic Acid on Proliferation

Cells of each cell line but SW 837 were seeded onto 96 well plates at a concentration of 6,000 cells/ml in 200 μl of RPMI-1640 medium/well, and cultured with and without 5 μM all-trans retinoic acid (Sigma, St. Louis, MO). MCF-7 breast carcinoma cells, which are known to express retinoic acid receptors and to be sensitive to all-trans retinoic acid treatment, were used as a positive control [33]. Fetal calf serum was omitted from the RPMI-1640 medium used for the assay. Media and ligand were changed every 24 hours. After 6 days of treatment, the cell number was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [34].

Northern Blot Analysis

Established human colorectal carcinoma cells were seeded onto 15 cm plates with 200,000 cells/plate and cultured until 90% confluent. The RPMI-1640 medium was changed and cells were incubated 48 hours without or with all-trans retinoic acid at a final concentration of 5 μM. Total RNA was isolated by the guanidinum thiocyanate-phenol-chloroform extraction [35]. Total RNA was isolated from both normal intestinal epithelial cells obtained from surgical specimens, colorectal carcinoma-derived cell lines, four primary colorectal carcinomas, and four liver metastases of colorectal carcinoma.

Approximately 15 μg of total RNA isolated from each sample was electrophoresed through 1% agarose gels containing 0.66 M formaldehyde, blotted overnight onto nylon membranes (Magna Graph, MSI, Westboro, MA) in 10 × SSC buffer, and baked for 2 hours at 80°C. Hybridization of RNA to the specific probes was performed with Denhardt's reagent and 50% Formamide [36]. Blots were exposed to X-ray film at −80°C for 1, 2, and 4 weeks.

The 0.83 kb cDNA probe for hRARα was obtained by digestion of the complete hRARα sequence by the re-

striction enzyme, Eco RI. The 0.6 kb and 0.9 kb cDNA probes for hRARβ were obtained by digestion of the complete hRARβ coding sequence by Eco RI/Bam HI. The cDNA probe for hRARγ was a full coding sequence of the gene. The 0.74 kb probe for hRXRα was obtained by digestion of the complete hRXRα coding sequence by Apa I/Hinc II. Probes were radiolabeled with ^{32}P-dATP by T7QuickPrime Kit (Pharmacia, Sweden). Specific radioactivity of each probe was about 2×10^9 cpm/mg DNA [36]. Quality and amount of the RNA was assessed by probing the blots with a cDNA for β-tubulin.

RT-PCR

Primers were prepared in the Department of Molecular Biology at the Cleveland Clinic Foundation and in the Center for Molecular Medicine, University of Oklahoma, and purified by high pressure liquid chromatography (HPLC) prior to use. First strand cDNA was prepared using Superscript II Reverse Transcriptase (Gibco BRL, Gaithersburg, MD) from total RNA. Briefly, 2 μg of total RNA in 2 μl of diethylpyrocarbonate (DEPC) water was mixed with 1 μl of 10 × DNase I buffer, 6 μl of DEPC-treated water, and 1 μl of DNase I. The mixture was digested (25°C, 15 minutes) to remove any genomic DNA contaminating the RNA samples. DNase I was inactivated (1 μl 20 mM EDTA, 65°C, 10 minutes) and 1 μl oligo $(dT)_{12–18}$ (Gibco BRL, 500 μg/ml) was added. Subsequently, the samples were heated (70°C, 10 minutes) and quick-chilled on ice. After centrifugation, 4 μl of 5 × First Strand Buffer (Gibco BRL), 2 μl 0.1 M dithiothreitol (DTT), 1 μl 10 mM deoxynucleotidetriphosphate (dNTP), and 1 μl Superscript II Reverse Transcriptase (Gibco BRL) were added. The mixture was incubated for 1 hour at 37°C.

The PCR reaction mixtures contained 10 μl reverse transcriptase reaction product, 10 μl 10 × PCR buffer (Gibco BRL), 1 μl 10 mM dNTP, 3 μl 50 mM $MgCl_2$, 5 μl each sense and antisense primer (10 mM), 1 μl Taq DNA polymerase (Gibco BRL, 5 U/ml), and sterile water to a final volume of 100 μl. PCR reaction mixtures were overlayed with 35 μl mineral oil in a 0.5 ml microfuge tube, then placed into a Perkin-Elmer thermocycler. PCR reaction products were separated by electrophoresis on a 2% agarose gel at 60 V for 2 hours, and the ethidium bromide-stained bands were visualized by UV transillumination.

Relative RT-PCR

This approach is based upon the modified protocol of the Arbitrarily Primed PCR [37, 38]. This technique yields relative concentrations of PCR products and allows the amplification of test and marker cDNAs each to be within the linear portion of the sigmoid PCR curve and for optimization of the reaction conditions without the need for a marker with the same concentration and similar physico-chemical sequence parameters as the test cDNA. This technique differs from ordinary RT-PCR described above and should not be considered strictly quantitative. The technique can be considered semi-quantitative and is not designed to measure absolute amounts of either mRNA or cDNA. First, 10 μg of total RNA isolated from SW 480 and SW 620 cells untreated and treated for 48 hours with 5 μM all-trans retinoic acid was dissolved in 10 μl of DEPC water, mixed with 1 μl of 10 × DNase buffer, and incubated with 1 μl DNase I (25°C, 15 minutes). DNase I was inactivated (1 μl 20 mM EDTA, 65°C, 10 minutes). RNA was extracted twice by phenol-chlorophorm and ethanol/2 M sodium acetate (22:1 v/v) precipitated. First strand cDNA was synthesized in the similar way as for regular PCR except that 100 ng of $p(N)_6$ random primer was used (Boehringer) per reaction per 5 μg of total RNA. After the reverse transcriptase reaction, the samples were heated up (95°C, 10 minutes), diluted up to 100 μl, and frozen at −20°C. To perform Relative RT-PCR, the first-strand cDNA samples to be compared were normalized simultaneously to the same β-Actin cDNA concentration by performing 20 cycles of PCR with β-Actin primers. Based upon the normalization, exactly the same amount of first strand cDNA was aliquoted from each sample, and 32 cycles of PCR were performed with each pair of primers. Normalization was checked each time by simultaneously amplifying in a separate tube for 20 cycles. The difference of gene expression between all-trans retinoic acid-treated and control cells was determined semi-quantitatively under ultraviolet (UV) light. The Relative RT-PCR was repeated using independent samples of total RNA. Table I contains the list of primers, the expected molecular weights of the RT-PCR products, and PCR conditions used. The identity of the RT-PCR products was confirmed by restriction enzyme digestion or sequencing.

Dot Blot ECL Assay With Murine Monoclonal Antibody Against hRARα

Normal human enterocytes, normal human colonocytes, colonic fibroblasts CD-Co 18, intestinal smooth muscle cells HISM, human colorectal carcinoma-derived cells, Colo 205, DLD 1, and HT 29 as well as surgical specimens of primary colorectal carcinomas, HCC 111, HCC 124, HCC 139 and surgical specimens of liver metastases of colorectal carcinomas, HCC 133LM, HCC 134LM, HCC 139 LM were suspended in 10 ml of buffer A [20 mM Tris-HCl, 1 mM EDTA, 0.6 M NaCl, pH 7.8 with protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM leupeptin, 1 mM antipain, 0.1 mM bacitracin, 1 TIU/ml aprotinin, 1 mM aminobenzamidine, 10 μM pepstatin A)] and washed twice. MCF-7 cells and hRARα-positive breast carcinoma were used as positive controls. Rat spleen was used as a negative control. After transfer to a glass homogenizer, each sample

TABLE I. List of Primers, PCR Conditions, and the Expected Molecular Weights of RT-PCR Products

Primer	Primer sequence	PCR conditions	MW (bp)
hRARα S	5′-TTG TCT GTC AGG ACA AGT CC	32 cycles (94°C, 60 s;	188
hRARα A	5′-CGT CTT CAC GAA ACT TCA CC	55°C, 60 s; 72°C, 120 s)	
hRARβ S	5′-TCG TCT GCC AGG ACA AAT CA	32 cycles (94°C, 60 s;	189
hRARβ A	5′-CGT CTT CAC GAA ACT TCA CC	55°C, 60 s; 72°C, 120 s)	
hRXRα S	5′-CAG GCA AGC ACT ATG GAG TGT	32 cycles (94°C, 60 s;	165
hRXRα A	5′-TG GCC AGG CAC TTC TGG TA	60°C, 60 s; 72°C, 120 s)	
hRXRβ S	5′-CTT TGT GCA ATC TGC GGG GAC	32 cycles (94°C, 60 s;	192
hRXRβ A	5′-TGG CCA GGC ACT TCT GAT ATC	60°C, 60 s; 72°C, 120 s)	
CRBP I S	5′-GTC GAC TTC ACT GGG TAC TGG A	32 cycles (94°C, 60 s;	441
CRBP I A	5′-TTG AAT ACT TGC TTG CAG ACC ACA	60°C, 60 s; 72°C, 120 s)	
CRABP I S	5′-CGG CAC CTG GAA GAT GCG CA	32 cycles (94°C, 60 s;	370
CRABP I A	5′-CCA CGT CAT CGG CGC CAA AC	60°C, 60 s; 72°C, 120 s)	
CRABP II S	5′-CCC AAC TTC TCT GGC AAC TGG A	32 cycles (94°C, 60 s;	410
CRABP II A	5′-CTC TCG GAC GTA GAC CCT GGT	60°C, 60 s; 72°C, 120 s)	
EGF-R S	5′-GT CTG CCG CAA ATT	33 cycles (94°C, 45 s;	544
EGF-R A	5′-CC GCG TAT GAT TTC	54°C, 45 s; 72°C, 90 s)	
MK S	5′-CTG CAA CTG GAA GAA GGA	32 cycles (94°C, 60 s;	206
MK A	5′-TTC CCT TCC CTT TCT TGG	60°C, 60 s; 72°C, 120 s)	
TG S	5′-GGG CGA ACC ACC TGA ACA AA	33 cycles (94°C, 45 s;	300
TG A	5′-GGT CAC TAC CTA GCA TGT TGT	54°C, 45 s; 72°C, 90 s)	
β-Actin S	5′-GCG CTC GTC GTC GAC AAC GGC	20 cycles, times and temperatures	220
β-Actin A	5′-CAT GGG GTA GCT CGT GCC GTA GCA GT	as for the primers tested	

S denotes sense sequence; A denotes antisense sequence.

was homogenized on ice in 2 ml of the buffer and centrifuged 15 minutes at 50,000 rpm and 4°C. Fat was removed from the sample and supernatant was transfered to the plastic tubes and the protein concentration was determined by Bio-Rad Protein Assay kit (BioRad, San Diego, CA). The plastic tubes with the supernatant were covered with gauze, frozen for 15 seconds in liquid nitrogen and lyophilized overnight at −60°C. After lyophilization, the protein concentration was equalized to 5.0 mg/ml, and 5 μl of the solution was mixed with 50 μl of distilled water. The entire volume was transfered onto nitrocellulose in a vacuum. Hybridization with murine anti hRARα monoclonal antibodies was performed using an ECL kit (Amersham, Little Chalfont, UK). The membrane was transfered to a plastic bag and exposed to Kodak film for 30, 60, and 120 minutes at room temperature.

Transfection of Chloramphenicol Acetyl Transferase Gene Under Control of Retinoic Acid Responsive Element Into SW480/SW620 Cells and CAT Assay

The pBLCAT2 reporter plasmid containing the chloramphenicol acetyltransferase (CAT) gene linked to a thymidine kinase promoter on the 5′ end to allow for a low basal activity and containing the SV40 polyadenylation signal attached to the 3′ end of the CAT gene to allow stabilization of the message was used as a control for the basal level of activity. The same plasmid containing the retinoic acid responsive element from the RARβ gene (pRARE-CAT) was used to test for functionality of the all-trans retinoic acid signaling pathway. Cells of each line were seeded onto twelve 10 cm plates with 50,000 cells/plate. Cells were cultured until 90% confluent. The RPMI-1640 medium containing 10% fetal calf serum was changed every 24 hours. The serum was analyzed by HPLC and confirmed to contain negligible amounts of vitamin A and all-trans retinoic acid. The plasmids were transfected by the calcium phosphate method with the glycerol shock [36]. Six plates of control cells were transfected with the pBLCAT2 plasmid. Effectiveness of the transfections was verified by PCR. After transfection, washing with PBS, and refeeding with medium, three plates were treated with 5 μM all-trans retinoic acid for 48 hours. Then, CAT activity was measured with a CAT ELISA kit (Boehringer-Mannheim, Indianapolis, IN) and compared to the CAT activity in the non-treated cells (Ratio C). Similarly, six plates of cells of the same line were transfected with the pRARE-CAT plasmid. Three of them were treated with the retinoid, and then CAT activity was measured, and compared to the CAT activity in the non-treated pRARE-CAT cells (Ratio R). Transformed uroepithelial cells HUC-PC and T24 were used as a negative control and a positive control for the transfection, respectively. The assay was performed in duplicates. The R/C ratio is the ratio of absorbances of ELISA for CAT activity in the absence and presence of 5 μM all-trans retinoic acid in cells transfected with the plasmid pRARE-CAT (R) to the ratio of absorbances of ELISA for CAT activity in the absence and presence of the hormone in cells transfected with the control plasmid pBLCAT2 (C). The R/C ratio close to 1 indicates that no significant induction of CAT gene occurred due to the

TABLE II. Results of MTT Assay for Antiproliferative Effect of 5 μM All-Trans Retinoic Acid (atRA) in Human Colorectal Carcinoma-Derived Cell Lines

Cell line	Control	S.D.	5 μM atRA	S.D.
Caco 2	2.71	0.33	2.56	0.19
Colo 205	*0.52*	*0.12*	*0.19*	*0.08*
DLD 1	1.67	0.29	1.53	0.72
HT 29	*1.72*	*0.26*	*0.32*	*0.16*
SW 480	*0.50*	*0.10*	*0.34*	*0.07*
SW 620	0.39	0.11	0.32	0.10
SW 1463	0.31	0.14	0.28	0.17
MCF-7	*1.41*	*0.05*	*0.62*	*0.04*

Results are the mean absorbances at 570 nm ± standard deviation from 32 wells. The italic type indicates the difference between control and test is statistically significant at $P < 0.05$.

interaction of the retinoid receptors with RARE of the plasmid under the retinoid treatment, and, therefore, the signaling pathway is not functional.

RESULTS

Effects of All-Trans Retinoic Acid on Proliferation

All cell lines studied grew well in RPMI 1640 medium supplemented with nonessential aminoacids and 10% fetal calf serum. All cell lines but two, grew well in the absence of serum; Colo 205 and SW 1463 grew much slower (data not shown) as compared to the cells cultured in medium with 10% fetal calf serum. Other than the HT-29 cells, all other lines exhibited the same morphology in the presence of serum or all-trans retinoic acid as in their absence. HT 29 cell morphology changed from the usual polygonal shape during the treatment with all-trans retinoic acid to a more rounded morphology and continued to exclude Trypan Blue.

Results of the MTT assay for the antiproliferative effect of all-trans retinoic acid are shown in Table II. A significant inhibition of proliferation ($P < 0.05$, Student's *t*-test) was observed in the populations of Colo 205 cells (threefold), HT 29 cells (fivefold) as compared to the non-treated cells. In addition, a statistically significant inhibition was observed in case of SW 480 cells (onefold). No significant difference was observed in case of Caco 2, DLD 1, SW 620, and SW 1463 cells.

Northern Blot Analysis and RT-PCR Study of Receptor mRNA Expression

Northern blot analysis identified hRARα mRNA as the expected 2.8 kb and 3.8 kb bands in total RNA isolated from cultured colorectal carcinoma cell lines (Fig.1). In two of the primary carcinomas and all four cases of colon cancer metastases to liver, bands corresponding to hRARα mRNA were not seen, even though the β-tubulin band was visible (Fig. 1). The more sensitive technique of RT-PCR with primers for hRARα ligand binding domain was applied with results shown in

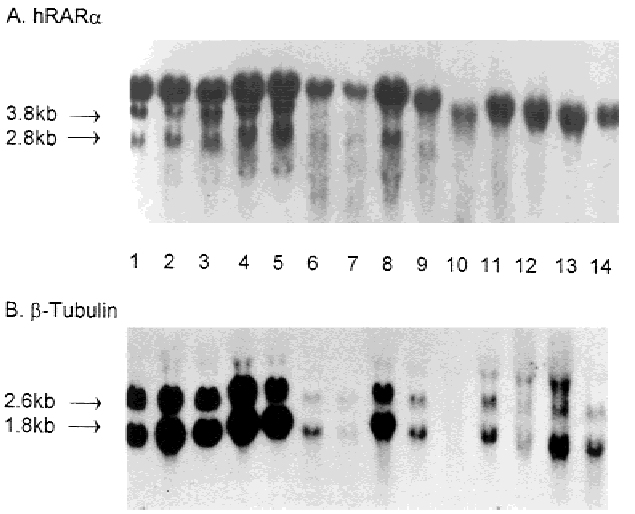

Fig. 1. **A:** Northern blot analysis of hRARα mRNA expression in colorectal carcinoma-derived cell lines, primary colorectal carcinomas, and liver metastases of colorectal carcinoma. Eco RI fragment of hRARα cDNA (0.83 kb) was used as a probe. **B:** Amounts of total RNA on the blot were normalized against β-tubulin cDNA. hRARα mRNA was expressed as 2.8 kb and 3.8 kb bands. Colorectal carcinoma-derived cells, lanes 1–5: Colo 205, DLD 1, HT 29, SW 620, SW 837; primary colorectal carcinomas, lanes 6–10: HCC 147, HCC 148, HCC 111, HCC 124, HCC 134; liver metastases of colorectal carcinomas, lanes 11–14: HCC 104LM, HCC 133LM, HCC 134LM, HCC 139 LM.

Figure 2A. In all instances, a single band corresponding to the expected 188 bp RT-PCR product of the hRARα ligand binding domain was present, including normal enterocytes and colonocytes, which were not shown on the Northern blot, as well as in the liver metastases that failed to show a band by Northern blot analysis. Because the RT-PCR method was more sensitive and was validated by the Northern blot analysis, it was used to investigate the presence of hRXRα transcripts, and in all instances, this gene was found to be expressed as well (Fig. 2B). A single band corresponding to the expected 165 bp product was seen in each sample. Northern blot analysis confirmed the presence of hRXRα transcripts at 5.6 kb (data not shown). Northern probing for hRARβ and hRARγ was inconclusive even at the longest exposure times.

hRARα Protein Expression

Figure 3 demonstrates the results of ECL analysis for the hRARα receptor protein. The densitometrically determined relative (to MCF-7) amounts of receptor protein are shown in Table III together with comparative amounts of mRNA determined densitometrically from the Northern blots. The range of mRNA amounts was similar for normal colonocytes and for established cell lines. Other than the DLD 1 line, the HT 29 and SW 620 lines contained almost the same amount of receptor protein as did normal colonocytes. In contrast, the primary

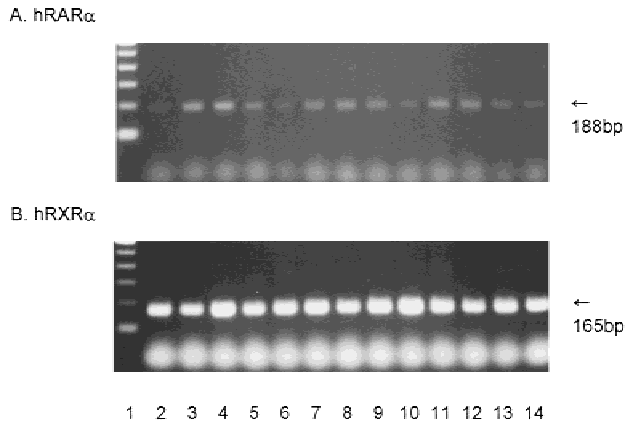

Fig. 2. Qualitative identification of hRARα **(A)** and hRXRα **(B)** cDNAs in normal colonocytes, colorectal carcinoma-derived cells, and liver metastases of colorectal carcinomas using primers for ligand binding domains of the receptors by ordinary RT-PCR. Identity of the products was confirmed by restriction enzyme digestion of the radiolabeled RT-PCR products and visualization on the 6% polyacrylamide gel. The first strand cDNAs were not normalized against β-Actin. Lane 1: molecular weight standards, 100 bp DNA ladder. Lane 2: normal human enterocytes (ileum). Lane 3: normal human colonocytes (sigmoid). Colorectal carcinoma-derived cells, lane 4: Caco 2; lane 5: Colo 205; lane 6: DLD-1; lane 7: HT 29; lane 8: SW 480; lane 9: SW 620; lane 10: SW 837; lane 11: SW 1463. Liver metastases of colorectal carcinoma, lane 12: HCC 133LM; lane 13: HCC 134LM; lane 14: HCC 139LM.

tumors and liver metastases contained up to 40-fold higher amounts of receptor protein. Comparison of receptor protein amounts in the sensitive HT 29 line and the insensitive DLD 1 and SW 620 lines shows that all the three had similar mRNA amounts, but the amount of receptor protein was roughly 10 times as high in the DLD 1 line as in the HT 29 and SW 620 lines. Of interest was the general lack of correlation between the amount of mRNA and the amount of receptor protein seen in the cells, even among the normal cells. This discrepancy was particularly striking for the liver metastases, in which the mRNA was not detectable by Northern blot analysis, but which corresponded to high amounts of receptor protein.

Relative Expression of Retinoid-Responsive Genes in SW 480/SW 620 Cell Lines

The results of Relative RT-PCR analysis of the SW 480 and SW 620 cell lines are summarized in Table IV. These two cell lines were derived from the primary tumor and a lymph node metastatic lesion of the same patient, but the SW 480 line was sensitive to all-trans retinoic acid while the SW 620 line was not. The two cell lines differed markedly in constitutive and stimulated expression of several of the retinoid responsive genes, selected gels of which are shown in Figure 4. In the presence of 5 μM all-trans retinoic acid, hRARα was down-regulated in the SW 480 cells but was marginally up-regulated in SW 620 cells (Fig. 4A). As found using Northern blot analysis in the other sensitive cell line, HT 29, hRARα mRNA was up-regulated as expected (data not shown). CRBP I was strongly up-regulated, as expected, in both cell lines (Fig. 4B). The MK angiogenic cytokine gene was sharply down-regulated in the SW 480 cells, but strongly up-regulated in the SW 620 cell line (Fig. 4C). EGF-R was twofold up-regulated in SW480 cells, but was constitutively low and further down-regulated in SW620 cells (Fig. 4D). The following genes were also measured with results listed in Table IV, but gels are not shown. The hRARβ gene was not present constitutively in either cell line, but was induced in both, as expected. CRABP I was down-regulated in the SW 480 cells as expected but up-regulated in SW 620. CRABP II was down-regulated about twofold in SW 480 cells, in contrast to the expected up-regulation. In the SW 620 cells, CRABP II was undetectable after treatment. TG gene was down-regulated in SW 480 cells, an unexpected finding, but was strongly up-regulated in SW 620 cells.

Plasmid Transfection and CAT Activity

Plasmid transfection was successful in all cases. As expected, the R/C ratio for HUC-PC cells, a negative control, was close to 1, indicating that the signaling pathway is not active. In contrast, the R/C ratio for T 24 cells denotes an almost twofold increase of CAT activity, indicating that the signaling pathway is functional. CAT activity in SW480 cells transfected with the plasmid pRARE-CAT was significantly increased (almost threefold) under treatment with 5 μM all-trans retinoic acid as compared to controls and SW 620 cells transfected with the same plasmid; the R/C ratio was 2.61. CAT activity in SW620 cells transfected with the pRARE-CAT plasmid remained in the same range as in the control cells transfected with the plasmid pBLCAT2; the R/C ratio was close to 1 (Table V).

DISCUSSION

Chemoprevention is an attractive option in cancer control because the risks of metastasis and cancer therapy are completely avoided. A number of factors have been tested as chemopreventive agents in colorectal tumorigenesis, including nonsteroidal anti-inflammatory drugs (aspirin, sulindac), difluoromethylornithine, calcium, vitamin D_3, and organoselenium compounds [39–42]. Although retinoids have been used successfully in animal studies to inhibit colorectal tumorigenesis, no clinical trials have been reported in humans. This study provides a molecular background for chemoprevention in human large intestine; however, we emphasize here that retinoid chemotherapy of advanced colorectal carcinomas will most likely be less successful. This is because a number of random molecular defects increases during tumor progression. As shown in the in vitro model, even genetically related tumor cells may differ significantly in response to the retinoid treatment, in functionality of the

Fig. 3. Results of ECL dot blot with murine monoclonal antibody against hRARα receptor protein. Lane 1: primary colorectal carcinoma HCC 139. Liver metastases of colorectal carcinomas, lanes 2–4: HCC 139LM, HCC 134LM, HCC 133LM. Lane 5: normal human enterocytes (ileum). Lane 6: normal human colonocytes (sigmoid). Lane 7: human colonic smooth muscle cells. Lane 8: human colonic fibroblasts. Colorectal carcinoma-derived cells, lanes 9–11: DLD-1, SW 620, HT 29. Primary colorectal carcinomas, lanes 12, 13: HCC 111, HCC 124. Positive controls, lane 14: MCF-7; lanes 15, 16: human primary breast carcinoma (dilutions 1:8 and 1:4). Negative control, lane 17: rat spleen.

TABLE III. Densitometric Analysis of hRARα mRNA and Receptor Protein

Sample	Relative amount of hRARα mRNA	Relative amount of the receptor protein
MCF-7 (positive control)	1.00	1.00
Normal cells		
Colonocytes	1.10	1.51
Smooth muscle cells	0.14	7.12
Colonic fibroblasts	0.09	0.06
Colorectal carcinoma-derived cells		
HT 29 (atRA-sensitive)	0.78	0.65
DLD 1 (atRA-insensitive)	0.72	7.01
SW 620 (atRA-insensitive)	0.81	0.75
Primary carcinomas		
HCC 111	2.42	26.64
HCC 124	0.18	34.98
HCC 139	ND	42.79
Liver metastases		
HCC 133 LM	ND	6.69
HCC 134 LM	ND	3.69
HCC 139 LM	ND	10.28

Results are expressed relative to amounts found in MCF-7 cells. ND denotes that mRNA was not detected by Northern blot analysis.
atRA = all-trans retinoic acid; hRARα = retinoic acid receptor type α.

pathway, or in the patterns of expression of retinoid-responsive genes. For the first time, we demonstrated that the key elements of all-trans retinoic acid signaling pathway are expressed in human intestinal epithelial cells, and that expression of the retinoid-responsive genes is modified whether the specific pathway is functional or not. In addition, results with cultured cells are compared with clinical specimens.

The antiproliferative assay showed that 3/7 of the cell lines were inhibited by 5 μM all-trans retinoic acid. This concentration is nonphysiological; however, no cytotoxic effect was observed. Such value remains within the average range of biologically active concentrations of the retinoid in in vitro cellular systems, i.e., between 1 μM and 10 μM [33, 43–47]. Thus, it appears that transformed intestinal epithelial cells tolerate the retinoid in a similar manner as the other transformed epithelial cell types do. Furthermore, according to Gudas (personal communication), with cancer cells, the antiproliferative effect of the retinoid may not occur at physiological concentrations, and to observe such effect in vitro, the extracellular concentration of the retinoid must be raised to distinctly nonphysiological levels. Indeed, one of the challenges of retinoid research is to discover retinoid compounds with less toxicity than all-trans retinoic acid so the dose can be increased sufficiently high to inhibit cancer or premalignant cells [43].

The major elements for all-trans retinoic acid signal transduction are hRARα, hRXRα, CRABP I, and CRABP II [1, 48]. As shown in Figures 1 and 2, hRARα and hRXRα mRNAs were expressed in all cells examined, and, in the case of hRARα, the protein was identified as well (Fig. 3). Thus, failure of some cell lines to show an antiproliferative response is not due to absence of these elements [49]. Interestingly, the observed modulation of expression of a number of retinoid-responsive genes under 5 μM all-trans retinoic acid stimulation suggests indirectly that signal transduction to the nucleus

TABLE IV. Summary of the Relative RT-PCR With Primers for Either Genes Encoding Elements of the All-Trans Retinoic Acid Signaling Pathway or Retinoid-Responsive Genes in SW 480/SW 620 Human Colorectal Carcinoma Model

		SW 480		SW 620	
Gene	Expected mRNA expression	Constitutive expression	atRA	Constitutive expression	atRA
hRARα [1]	Up	High	Fivefold down	Medium	Onefold up
hRARβ	Up	Absent	Present	Absent	Present
hRXRα [1]	No change	High	No change	High	No change
hRXRβ	No change	High	No change	High	Twofold down
CRBP I [1]	Up	Medium	Eightfold up	Low	Threefold up
CRABP I [1]	Down	Moderate	Threefold down	Moderate	Threefold up
CRABP II [1]	Up	Moderate	Twofold down	Moderate	Absent
EGF-R [1,28]	Down	High	Twofold up	Low	Twofold down
TG [29]	Up	High	Fivefold down	High	Fivefold up
MK [30]	Up	High	Fivefold down	High	Fivefold up

The expected mRNA expression under all-trans retinoic acid stimulation is based on the published experimental data [1, 28–30].
atRA = all-trans retinoic acid; RT-PCR = Reverse transcriptase polymerase chain reaction (see footnote to Table I).

occurs (Table IV), whether cells are sensitive to the antiproliferative action of the retinoid or not, and whether the appropriate signaling pathway is functional or not. Indeed, the plasmid transfection study demonstrates that all-trans retinoic acid signaling pathway is functional in SW480 cells; however, it is not active in SW620 cells. Although molecular defects responsible for lack of functionality of the pathway in the latter case are unknown (no mutations in hRARα gene were found in both cell lines; data unpublished), we suppose that modulation of expression of retinoid-responsive genes in SW620 cells occurs due to the complex links of the retinoid pathway with the other pathways through the common molecular elements.

Previous investigators have speculated that high expression of CRABP I protein and non-inducibility of the hRARα gene were involved in insensitivity to retinoids [1, 48]. In our study, CRABP I mRNA expression was increased in the insensitive SW 620 cell line and decreased in the sensitive SW 480 cells following all-trans retinoic acid stimulation. Elevations in expression of the CRABP I gene or in protein amounts may serve as a useful marker to exclude patients resistant to retinoid chemoprevention by the mechanism of retinoid sequestration by CRABP I. The hRARα gene does not seem to be involved with all-trans retinoic acid resistance or sensitivity. Indeed, the hRARα mRNA was down-regulated fivefold in the sensitive SW480 cells, an unexpected finding given that the gene contains a retinoid-responsive element [50], but was up-regulated as expected in the sensitive HT 29 line [51]. Additionally, the SW 620 cells, which are resistant, also showed weak up-regulation of hRARα mRNA expression. Thus, no consistent pattern of expression of retinoid-responsive genes is associated with resistance or sensitivity [51, 52].

A number of other interesting and unexpected differences in expression of the retinoid-responsive genes was reported in Table IV. On the basis of previous reports, CRABP II was expected to be up-regulated by all-trans retinoic acid treatment, but was found to be twofold down-regulated in the SW 480 cells and was undetectable in the SW 620 cells. Also in contrast to previous reports with cell lines derived from lung and squamous cell carcinoma [1], hRARβ was induced in the SW 480 and SW 620 cell lines [51], although it was not found in untreated cells by either RT-PCR or Northern blot analysis. Transglutaminase has a retinoid-responsive element and has been reported to be up-regulated by all-trans retinoic acid [29], which it was in SW 620 cells, but it was unexpectedly sharply down-regulated in the SW 480 cells. MK angiogenic cytokine, which is all-trans retinoic acid responsive in the absence of an identifiable retinoid-responsive element [30], was sharply up-regulated in the metastatic SW 620 cells but unexpectedly strongly down-regulated in the SW 480 cells. EGF-R is an element in an important cellular signaling pathway linked to all-trans retinoic acid signaling and is down-regulated by the retinoid in human ME 180 epidermoid carcinoma cells [28]. In the SW 480 cells, the constitutive level of expression was unexpectedly high and was weakly up-regulated, but in the SW 620 cells, the constitutive expression was very low and was further down-regulated by the retinoid.

A number of potential mechanisms could be operating during intestinal tumorigenesis to produce resistance to all-trans retinoic acid in tumor cells. The resistance may arise either by altering genes involved in signal transduction, by mutating some of retinoid-responsive genes, or by altering the way the retinoid signaling interacts with elements of the other signaling pathways and metabolic

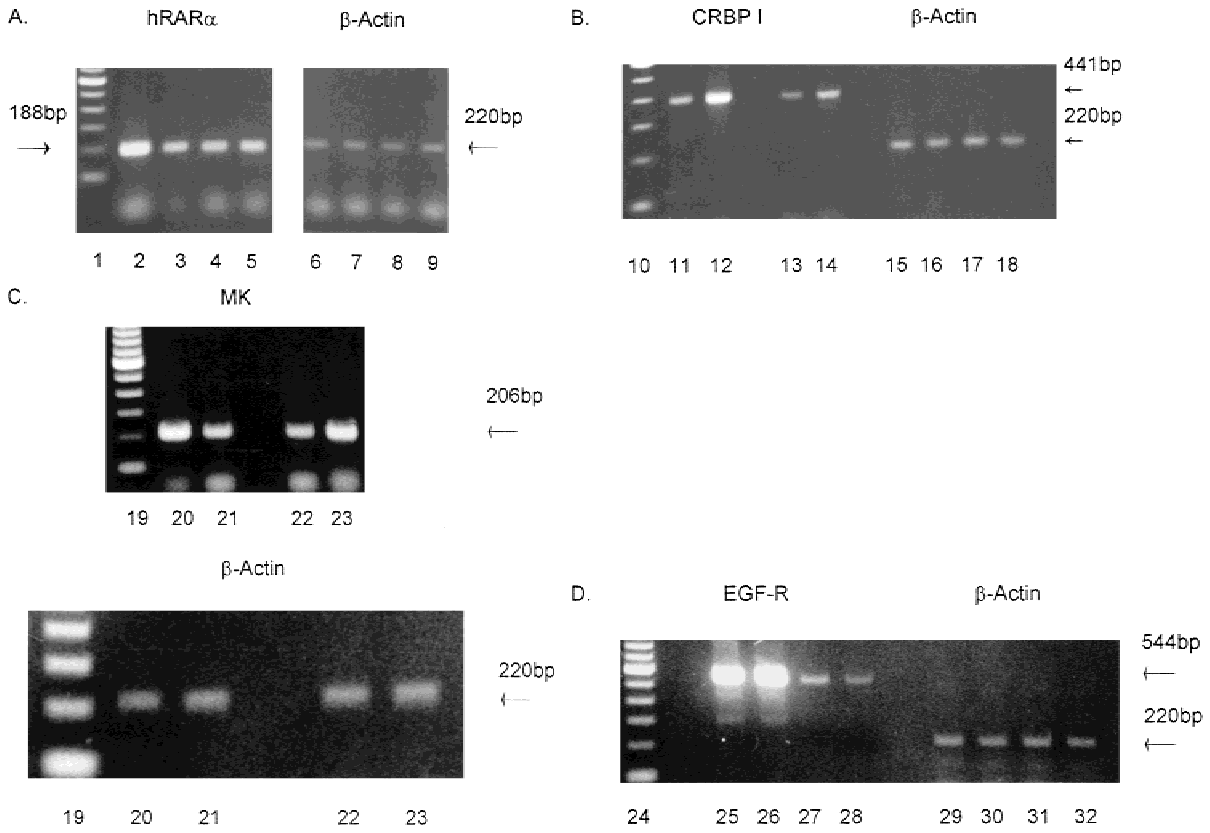

Fig. 4. Relative RT-PCR for hRARα **(A),** CRBP I **(B),** MK cytokine **(C),** and EGF-R **(D)** mRNA in SW 480/SW 620 cells treated with and without 5 μM all-trans retinoic acid for 48 hours. Total RNA was isolated and first strand cDNA was normalized against β-Actin. The analysis was repeated twice independently. **A:** hRARα, lane 1: molecular weight marker, 100 bp DNA ladder; lane 2: SW 480 control; lane 3: SW 480 all-trans retinoic acid (atRA)-treated; lane 4: SW 620 control; lane 5: SW 620 atRA-treated. Normalized β-Actin, lanes 6–9. **B:** CRBP I, lane 10: molecular weight marker, 100 bp DNA ladder; lane 11: SW 480 control; lane 12: SW 480 atRA-treated; lane 13: SW 620 control; lane 14: SW 620 atRA-treated. Normalized β-Actin, lanes 15–18. **C:** MK cytokine (top) and normalized β-Actin (bottom). Lane 19: molecular weight marker, 100 bp DNA ladder. Lane 20: SW 480 control. Lane 21: SW 480 atRA-treated. Lane 22: SW 620 control. Lane 23: SW 620 atRA-treated. **D:** EGF-R, lane 24: molecular weight marker, 100 bp DNA ladder; lane 25: SW 480 control; lane 26: SW 480 atRA-treated; lane 27: SW 620 control; lane 28: SW 620 atRA-treated. Normalized β-Actin, lanes 29–32.

processes. As shown in Table III, no simple correlation existed among hRARα mRNA expression, receptor protein amount and all-trans retinoic acid sensitivity among the cancer cells, or between amounts of the mRNA and the receptor protein in the normal intestinal epithelial cells. A similar lack of correlation was reported previously for other genes in normal intestinal epithelial cells, where regulation of gene expression occurred through regulation of mRNA lifetime, transport, translation, and protein turnover [53, 54]. The extremely high amounts of hRARα receptor protein found in the primary tumors, metastatic tumors and the DLD 1 cell line suggest uncoupling of these regulatory mechanisms and may reflect alterations associated with tumorigenesis.

Molecular characterization of the closely related SW 480 and SW 620 cell lines, representing consecutive stages of tumor progression, points out that expression of a number of genes normally acting as elements in retinoid signaling or linked to the pathway, for example EGF-R (Table IV) and RNase L [55], is functionally altered. In the case of RNase L, expression is completely shut off, even though the gene is present structurally unaltered in the genome [55]. Similarly, constitutive expression of EGF-R mRNA in SW 620 cells is sharply lower than in SW 480 cells. This low expression in SW620 cells was not due to a mutation of the gene that would be detected by PCR/SSCP (data unpublished; also Chakrabarty, personal communication). Although neither gene can be considered a classic tumor suppressor gene, the observed alterations in gene transcription may illustrate an additional mechanism operating in tumor progression. As shown by similar findings for ABO gene transcripts in human bladder tumors, inhibition of gene expression to modulate function of certain pathways may represent a general phenomenon in tumorigenesis [56].

The resistance of tumor cells can also be studied from a holistic perspective. Then, the functional status of a cell depends not so much on the activity of a single gene or even a set of genes (e.g. the retinoid-responsive genes), but rather upon the conjugated activities of the whole

TABLE V. Functionality of All-Trans Retinoic Acid Signaling Pathway as Tested With Cells Transfected With Plasmid pBLCAT2 (Control) and Plasmid pRARE-CAT Containing Chloramphenicol Acetyltransferase Gene (CAT) Under Control of Retinoid Responsive Element (RARE)

Cells	pBLCAT2 Control	pBLCAT2 atRA	pRARE-CAT Control	pRARE-CAT atRA	R/C
Control uroepithelial cells					
HUC-PC	0.335 ± 0.039	0.341 ± 0.013	0.407 ± 0.019	0.381 ± 0.043	0.92
T 24	0.341 ± 0.020	0.373 ± 0.045	0.364 ± 0.021	0.771 ± 0.097	1.94
Colorectal carcinoma-derived cells					
SW 480	0.333 ± 0.014	0.334 ± 0.010	0.349 ± 0.016	0.910 ± 0.032	2.61
SW 620	0.307 ± 0.016	0.313 ± 0.008	0.304 ± 0.011	0.322 ± 0.020	1.04

The R/C ratio is the ratio of absorbances of ELISA for CAT activity in the absence (control) and presence (atRA) of 5 μM all-trans retinoic acid in cells transfected with the plasmid pRARE-CAT (R) to the ratio of absorbances of ELISA for CAT activity in the absense (control) and presence (atRA) of 5 μM all-trans retinoic acid in cells transfected with the control plasmid pBLCAT2 (C). The R/C ratio close to 1 indicates that no significant induction of CAT gene occurred due to the interaction of the retinoid receptors with RARE of the plasmid under the retinoid treatment, and, therefore, the signaling pathway is not functional.

A symbol ± denotes standard deviation.

atRA = all-trans retinoic acid; pBLCAT2 = plasmid containing the CAT sequence without the RARE element; pRARE-CAT = reporter plasmid containing the retinoic acid responsive element from the RARβ gene.

network of genes, mRNAs, and proteins functioning according to the rules of complexity and probabilistic associations [57, 58]. In consequence, cancer cells are not unregulated [59]; they may even follow normal rules of regulation. However, the altered network produce easily uncoupling between a microscopic-scale effect such as expression of the retinoid-responsive genes and a macroscopic-scale effect such as antiproliferative action of the retinoid. In other words, due to complexity of the network, cellular response to the action of the retinoid cannot be predicted on the basis of the pattern of gene expression [52] and resistance may occur irrespective of the presence of functional retinoid receptors and signaling pathway [49]. Then, the resistance would not be the exclusive feature of cancer cells. Under certain circumstances, it might appear in a population of normal cells.

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